Exhibit B

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Effects of dynamic fluid activity from an electric toothbrush on in vitro oral biofilms

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Abstract

Objectives: To determine the plaque-removing ability of a Sonicare Plus® electric toothbrush in an in vitro model.

Material and Methods: Multispecies oral biofilms derived from human saliva were grown on hydroxyapatite discs in a constant-depth film fermenter. The biofilms were placed in a typodont model so that they mimicked the interproximal plaque between teeth 46 and 47 and were then treated with an electric toothbrush, both activated and inactivated. The distance from the bristle tips to the edge of the disc was 2.65 mm. Brushing action was controlled by a specially constructed brushing machine. After brushing, the number of viable bacteria removed from, and remaining in, the biofilms were determined.

Results: In all, 73.70% of viable bacteria in the biofilms were dislodged from the discs using the activated toothbrush. An inactivated toothbrush removed only 3.66%. Scanning electron microscopy and confocal microscopy revealed differences between untreated and treated biofilms.

Conclusion: The fluid shear forces generated by the electric toothbrush penetrated at least 2.65 mm beyond the reach of the bristles and these forces contributed to the toothbrush's plaque-removal ability ($p < 0.001$).

Key words: biofilm; plaque; sonic toothbrush; constant-depth film fermenter

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Dental plaque is a biofilm that forms at the solid–liquid interface between the tooth and saliva (Wilson, 2001). Dental caries is a plaque-associated disease characterised by a progressive disintegration of the tooth structure (Kidd & Joyston-Bechal 1997). Caries prevention is achieved by the removal of plaque by brushing and a number of electric devices are now available for this purpose (Fischman 1997). Electric toothbrushes commonly consist of a vibrating and/or oscillating brush head. There is evidence to suggest that plaque removal by electric toothbrushes is better than that achieved by manual brushes (van der Weijden 1998) and that, by regular use, electric toothbrushes can significantly improve periodontal health (Ho & Niederman 1997, Robinson et al. 1997).

An electric toothbrush (Sonicare Plus®) has been developed by Philips Oral Healthcare (Snoqualmie, USA), which operates at a frequency of 260 Hz and removes plaque by generating localised hydrodynamic shear forces (Khambay & Walmsley 1995). Microelectronics within the brush handle produce a rapidly oscillating magnetic field that induces an oscillation in the brush head. The frequency and amplitude of oscillation produces a bristle tip velocity that, when inserted in a fluid/air environment, creates turbulent fluid and bubble activity and associated shear forces. In vivo studies have shown the superior plaque-removing ability of this brush compared with manual brushing (Tritten & Armitage 1996, Stanford et al. 1997). However, confounding factors with in vivo plaque-removal studies are the poor reproducibility of results, the inability to standardise treatment and variations in the human oral microflora. Patients involved in any form of dental regime tend to improve their oral hygiene; additionally, such studies are often conducted in academic research institutions where the ''dental IQ'' of patients is relatively high (Overholser 1988). A procedure, which has the advantage of the reproducibility of an in vitro model system married to the realism of in vivo plaque, was therefore developed for examining the plaque-removal efficacy of an electric toothbrush. Both activated and inactivated electric toothbrushes can be studied to investigate the ability of fluid motion generated by an activated toothbrush's bristles to remove plaque in the absence of direct bristle contact.

The purpose of this study was to determine and quantify the effects of the fluid activity, beyond the bristles, delivered by an electric toothbrush on microcosm dental plaques grown on hydroxyapatite (HA) discs in a con-

stant-depth film fermenter (CDFF) under conditions similar to those that would exist in vivo.

Material and Methods

Preparation of CDFF

Oral biofilms were grown in a CDFF (University of Wales, Cardiff, UK). The CDFF consists of a rotating turntable holding 15 polytetrafluoroethylene (PTFE) pans, each pan containing five cylindrical holes filled by PTFE plugs. The CDFF was loaded with 75 5 mm-diameter HA discs (Clarkson Chromatography Products, South Williamsport, PA, USA), which were inserted on top of the PTFE plugs and recessed to a depth of 200 μm before autoclaving at 121°C for 30 min. Following inoculation, nutrient fluid was dripped onto the turntable, spread over the discs and removed by the action of scraper blades giving biofilms of a fixed depth. The modus operandi for the CDFF (Peters & Wimpenny 1988) has undergone an evolution with experience at the Eastman Dental Institute, specifically for growing models of dental plaque (Wilson et al. 1995, Pratten & Wilson 1999, Pratten et al. 1998b, Roberts et al. 2001).

Inoculation of the CDFF

Saliva was collected from 20 individuals (aged 20–40 years, in good oral health, a mixture of nonsmokers and smokers) in sterile containers. Five millitres of phosphate-buffered saline (PBS) was added to each of the samples and these were pooled. Glycerol (BDH Chemicals, Poole, UK) was added to 15% volume and the mixture was divided as 2 ml aliquots in cryo-vials and stored at −80°C. For each fermenter run, a thawed 2 ml aliquot was aseptically added to a flask containing 1 l of an artificial saliva containing hog gastric mucin, without urea (Pratten et al. 1998a) at 37°C. The inoculum was then pumped into the sterile CDFF at a rate of 0.72 l/day. Artificial saliva medium flow was then started the following day at 0.72 l/day, the mean resting saliva flow rate in humans (Guyton & Hall 1997, Lamb et al. 1991).

Biofilm growth and sampling

After at least 8 days growth, three sampling pans were removed from the CDFF and 10 of the 15 discs were used for treatment with a Sonicare Plus®. The operation of the CDFF demands that the PTFE pans are removed sequentially, so sample randomisation was incorporated by requesting impartial colleagues, blinded as to the subsequent treatment, to select biofilms.

Biofilm challenging with Sonicare Plus®

A pair of HA discs were carefully dipped into 1 ml of sterile PBS to wash off cells present in the liquid phase. The discs were then inserted into recesses located in plastic teeth (Fig. 1c) designed to simulate interproximal plaque between teeth 46 and 47. The teeth containing the discs were then placed in an exposure chamber (Fig. 1d) mounted on a specially constructed brushing machine (Fig. 2). The exposure chamber was filled with 7 ml PBS containing 0.8 g/l gastric mucin. Using an ''activated'' brush (i.e. with its electronically induced vibrating action operating), a reciprocating brushing action of 0.26 Hz was initiated with the brush at an angle of 40° with a horizontal and vertical load of 62 g (±5 g; 88 g vector total) between the brush and teeth; this was in accordance with the manufacturer's data associated with typical use. The distance travelled by the brush during one brushing cycle was 9.5 mm and the duration of brushing (exposure time) was 15 s. The above procedure was repeated but with the brush in an ''inactivated'' condition (i.e. with its electronically induced vibrating action not operating).

In order to document the separation between the discs and the bristles of the brush when activated, the teeth were marked with lipstick, which could easily be removed when contacted by the moving bristles (Figs. 1a and b). The brushing machine was set up as described previously, but without PBS in the sample holder, because the aim was to determine whether there was any contact between the bristles and the discs rather than the extent of any fluid shear activity. The experiment was conducted five times, returning ten bristle/disc separation values.

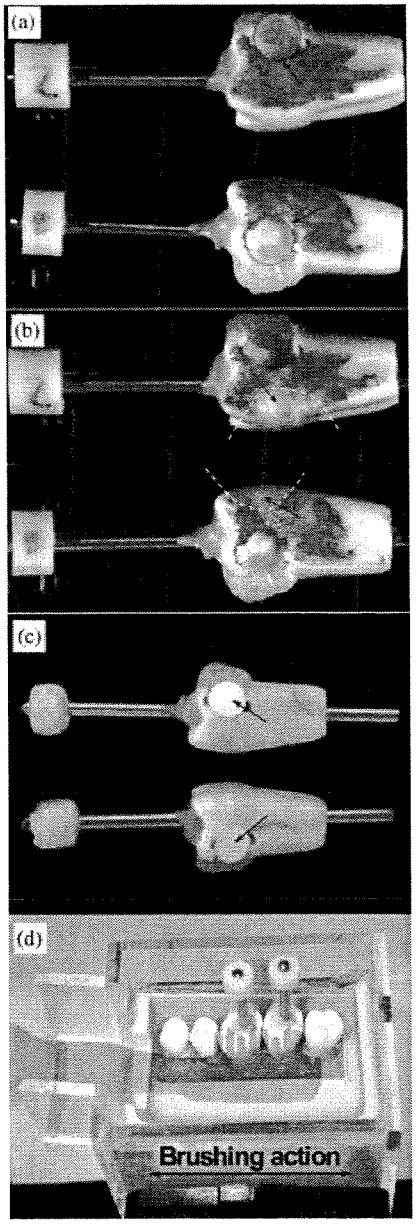


Fig. 1. (a) Model teeth covered in a standard cosmetic lipstick. The recesses for holding the 5 mm HA discs are shown by the arrows. (b) Model teeth after brushing by an active Sonicare Plus without a liquid medium present to measure the separation between the disc recesses and the zone of contact by the bristles (bounded by the dashed line). The mean separation was measured at 2.65 mm. (c) Model teeth with the 5 mm HA discs located in the recesses. The surface of the HA discs are flush with the contours of the teeth. (d) Model teeth located in the exposure chamber. The two HA discs were adjacent to each other to represent the site of interproximal plaque. The approximate position of the brush is (shown as a shadow) for illustrative purposes only. The direction of the 9.5 mm brushing action is perpendicular to the biofilm-coated surfaces of the HA discs.

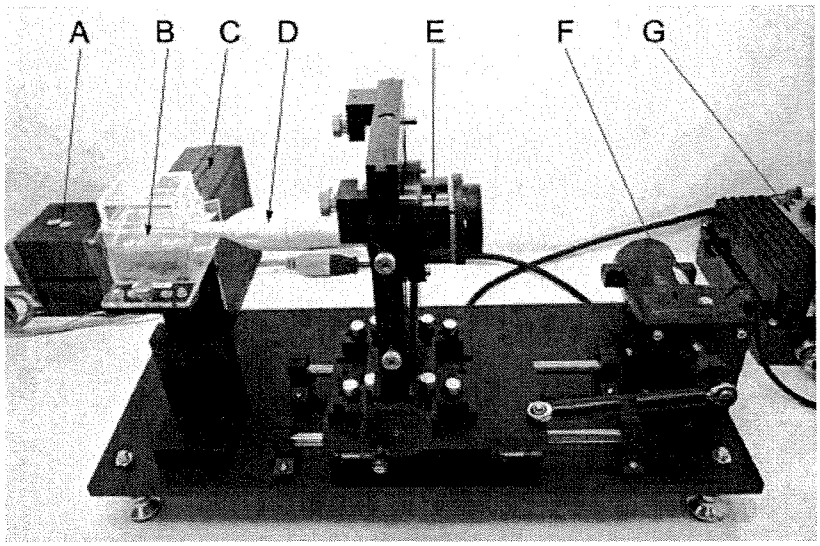


Fig. 2. Brushing machine: (A) transformer; (B) exposure chamber; (C) load cell, for measuring the vertical and horizontal load between the brush and the exposure chamber (teeth and typodont); (D) Sonicare Plus® toothbrush; (E) toothbrush holder, containing a mechanism for the fine adjustment of the position of the toothbrush to achieve the correct loads and position of the brush head against the gingival margin; (F) electric motor with eccentric cam, for generating the 9.5 mm linear displacement of the toothbrush holder; (G) motor speed controller, normally set at 0.26 Hz (four cycles during the 15 s exposure).

Posttreatment sampling

After the brushing, 5 ml of the suspension was transferred from the sample holder into a sterile container with glass beads. The samples were vortex-mixed for 30 s to break up bacterial clumps and serial dilutions prepared in PBS.

To determine the number of viable cells remaining on the discs after treatment, these were placed into 7 ml of sterile PBS containing glass beads, vortex-mixed for 30 s and serial dilutions prepared in sterile PBS. The discs were examined by scanning electron microscopy (SEM) to determine whether all adherent bacteria were removed by vortex mixing. Aliquots of the dilutions were plated in quadruplicate onto blood agar (BA) (Becton Dickinson, Franklin Lakes, NJ, USA) for total anaerobic counts, Mitis salivarius agar (MSA) (Difco Laboratories, Detroit, MI, USA) for *Streptococcus* species, *Veillonella* agar (VA) (Difco) for the isolation of *Veillonella* species and cadmium fluoride-acriflavin-tellurite (CFAT) agar (Zylber & Jordan 1982) for the isolation of *Actinomyces* species. All plates were incubated anaerobically at 37°C for 48 h and the resulting colonies counted.

Two other discs were brushed with the electronic effects of the Sonicare inactivated while another two were only dipped in sterile PBS before microscopic examination by either SEM or confocal laser scanning microscopy (CLSM) using LIVE/DEAD® *Ba*cLight™ staining (Molecular Probes, Eugene, OR, USA) to differentiate between viable and nonviable cells on the basis of membrane integrity.

Statistical analysis

Where appropriate, confidence intervals were calculated at 95% to give a range of values, consistent with the data, that is believed to encompass the actual or ''true'' population value. *p*-Values were calculated to measure the significance of the statistical evidence in favour of the null hypothesis (the activated and inactivated toothbrushes remove the same amount of bacteria).

Results

Figs. 1a and b show the results of a typical experiment to determine how close the brush bristles came to the hydroxyapatite discs. It can be seen in Fig. 1b that some of the lipstick was removed from the teeth and that this zone did not make contact with the recess holding the disc. The distance between the edge of the brushed zone and the closest edge of the disc recess was 2.65 mm (2.597–2.703 mm with 95% confidence intervals).

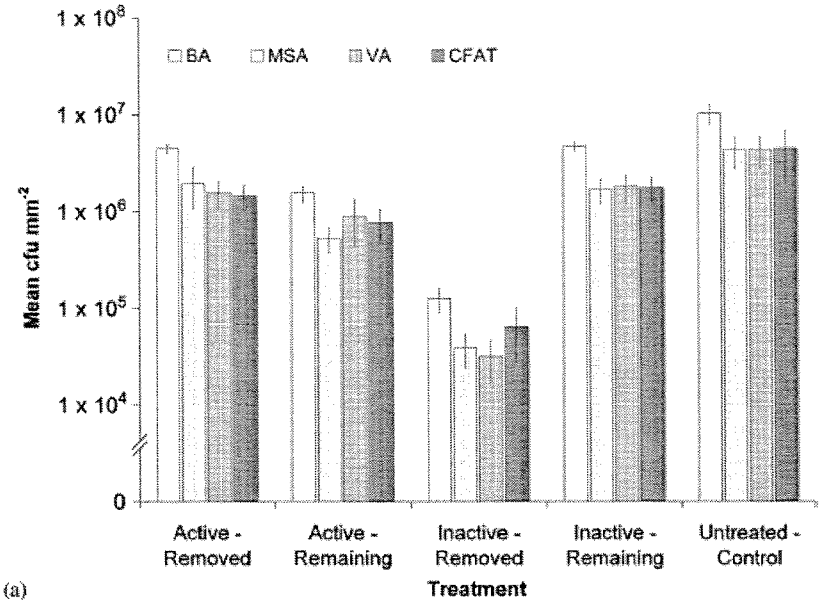
The proportions of the different bacterial species present in the biofilms (measured as viable counts on the selective agars) were 32.82% *Streptococcus* spp., 33.20% *Veillonella* spp. and 33.98% *Actinomyces* spp. The average density of bacteria in the biofilms was 1.03×10^7 CFU/mm^2 (6.10×10^6 to 1.45×10^7 CFU/mm^2 with 95% confidence intervals).

SEM of untreated biofilms showed structures typically found in in vivo plaques such as chains of streptococci (Figs. 4a and c) and ''corn-cobs''. CLSM of the biofilms showed a complex structure with chains of streptococci and water channels. Vital staining of the biofilms indicated that the majority of the live bacteria present were cocci.

The data obtained from the series of nine experiments (quadruplicate counts taken from each experiment, $n = 31$–36) are summarised in Figs. 3a and b. These show that the total number of viable bacteria removed (in terms of viable counts on blood agar) by an activated toothbrush was 4.49×10^6 CFU/mm^2 (3.59×10^6 to 5.39×10^6 with 95% confidence intervals) while an inactivated brush removed only 1.27×10^5 CFU/mm^2 (7.27×10^4 to 1.81×10^5 with 95% confidence intervals). The activated toothbrush removed significantly ($p < 0.001$) more bacteria than the inactivated brush for all bacteria counted on the different selective media.

The percentage of bacteria removed (based upon the sum of the number of bacteria removed and the number of bacteria remaining on the HA discs) by an activated toothbrush was 73.70% (57.70–89.7% with 95% confidence intervals), and the percentage of bacteria removed by an inactivated toothbrush was 3.66% (0.48–6.84% with 95% confidence intervals). The activated toothbrush removed a significantly ($p < 0.001$) higher percentage of bacteria than the inactivated brush for all bacteria counted on the different selective media.

CLSM images of untreated biofilms showed few nonviable cells (Figs. 4e and f). Treated biofilms contained only small amounts of residual biofilm and these fragments were viewed by CLSM. This showed that the viable cells (green channel) were mostly cocci (Fig. 4g); conversely, the dead cells were predominantly filamentous (red channel) (Fig. 4h).



(a)

Biofilm Removed (%)	Selective Media Type BA	MSA	VA	CFAT
Sonicare Active	73.70 ± 16.00	72.86 ± 20.25	71.29 ± 22.00	69.10 ± 22.26
Sonicare Inactive	3.66 ± 3.18	9.33 ± 11.47	7.88 ± 9.65	8.83 ± 11.69

(b)

Fig. 3. (a) Number of CFU/mm^2 of biofilm for different samples obtained from the brushing experiments, bacteria removed and remaining on the HA disc with active or inactive brushes on four different media: blood agar (BA) nonselective growth, *Mitis salivarius* agar (MSA) for the selective growth of *Streptococcus* species, *Veillonella* agar (VA) for the selective growth of *Veillonella* species and cadmium fluoride-acriflavin-tellurite (CFAT) agar for the selective growth of *Actinomyces* species. Error bars represent confidence intervals at 95%. The *p*-values associated with a comparison between active and inactive brushes (for both the number of bacteria removed and the number of bacteria remaining) were significant in every case (i.e. $p = 0.001$ or less). (b) Table showing the percentage of biofilm removed from HA discs by active and inactive brushes. These percentages were calculated based upon the sum of the numbers of bacteria removed and the numbers of bacteria remaining. The results of the four selective media types were significant in each case (i.e. $p = 0.001$ or less).

Discussion

Numerous studies have used the CDFF to grow biofilms with a composition and structure similar to those found in supragingival dental plaque in vivo. The microcosm plaques grown in this series of experiments were similar in composition to those found in approximal dental plaques in vivo (Marsh & Martin 1999). Prior to treatment, typical plaque structures such as chains of streptococci and ''corn-cobs'' were visible by SEM. CLSM of the biofilms showed the presence of water channels and ''stacks'' typical of many biofilms and previously found in oral biofilms grown in vitro and in vivo (Pratten et al. 2000, Wood et al. 2000). After treatment with an activated toothbrush, very few chains of streptococci could be seen in the remaining biofilm; however, many individual cocci were still evident as were an increased number of filamentous morphotypes.

With regard to the removal of biofilms from the discs, the design of the mouth model and brushing machine used enabled the reproducible determination of the efficacy of a particular brushing regime in removing interproximal plaque. The presence of a mucin-containing fluid in the mouth model simulates the saliva present in vivo. This represents a better simulation of the in vivo situation than liquid-free models that have been used previously (Driesen et al. 1998).

A considerably greater proportion of the biofilm was removed from the hydroxyapatite discs when the activated (73.70%) rather than the inactivated (3.66%) toothbrush was used. Biofilm removal from HA discs treated with an activated toothbrush tended to exhibit a definite ''cleared zone'' proximal to the bristles. SEM images of the residual biofilms further away from these cleared zones showed few streptococcal chains with a visibly increased proportion of filamentous bacteria. Typical SEM images showed the biofilm to have an upper layer of streptococci with lower layers of filamentous bacteria (*Actinomyces* species and a number of spirochaete morphotypes). The upper layer of the biofilm was often cracked by SEM sample preparation, further revealing the details of the layered differentiation. It is suggested that the liquid shear forces generated by the sonic toothbrush affect, for the most part, the outer layers of dental plaque.

Statistical analysis of counts obtained from the selective agars elude to the preferential removal of *Streptococcus* species over *Actinomyces* species ($p = 0.183$) by Sonicare Plus® and fewer streptococci remaining in the biofilm after brushing than *Veillonella* species ($p = 0.110$) or *Actinomyces* species ($p = 0.095$). While not *statistically* significant, these data support the SEM observation of the preferential removal of streptococci.

Biofilms treated with an inactivated brush were relatively unaffected with very few viable bacteria removed from the discs. This is because the bristles of the brush did not make contact with the biofilms at any point during the experiment and there was insufficient kinetic energy in the brushing action alone to generate the fluid shear forces necessary to remove a significant number of bacteria beyond the reach of the bristles.

The disparity in cell viability between streptococcal chains and filamentous bacteria, as revealed by viable staining, after brushing suggests that spherical bacteria are more resilient than filamentous bacteria to the liquid shear forces generated by the brush. The vulnerability of filamentous organisms to liquid shear is likely to be due to a (relatively) long filament crossing a range of shear gradients. This would also explain the breaking up of streptococcal chains into their component cocci in treated samples.

In conclusion, the amount of biofilm removed (in terms of viable bacteria) by an activated Sonicare Plus® was 20

times greater than that removed by an inactivated brush. This implies that the fluid shear effects induced by the oscillation of the brush head significantly contribute to the plaque-removing ability beyond the bristles. The fluid shear forces induced by the activated toothbrush are of sufficient magnitude to remove oral biofilms from a distance of at least 2.65 mm from the bristles.

Acknowledgments

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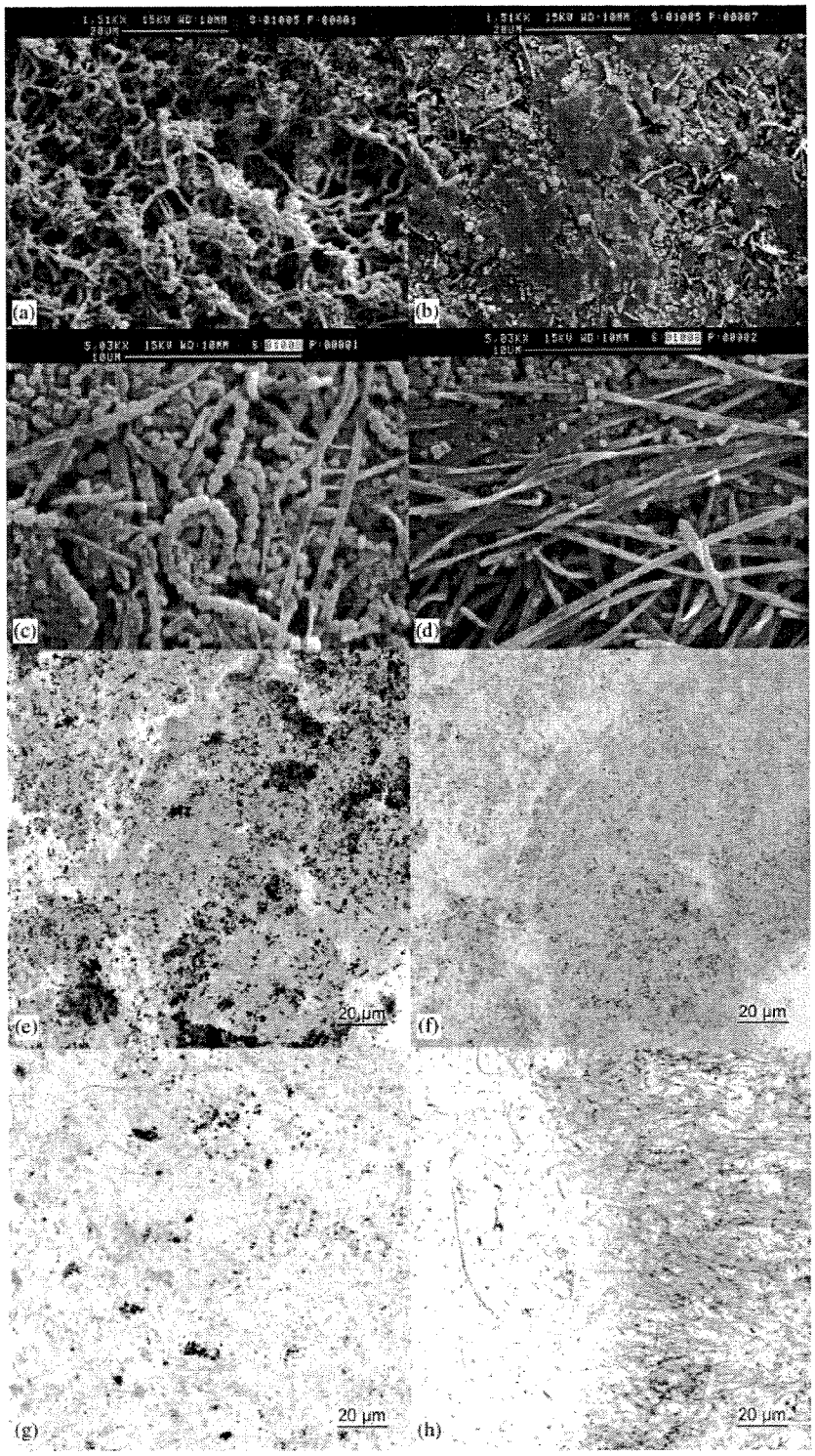

Zusammenfassung

Einfluss der dynamischen Flüssigkeitsaktivität einer elektrischen Zahnbürste auf orale Biofilme in vitro

Zielsetzung: Untersuchung der Fähigkeit der elektrischen Zahnbürste Sonicare Plus® zur Plaqueentfernung in einem *In-vitro*-Modell.

Fig. 4. Microscopic analysis of treated and untreated biofilms: (a–d) scanning electron micrographs; (e, f) confocal laser scanning micrographs (shown as negatives for clarity). (a) SEM (× 1510) of plaque biofilm grown in a CDFF, on a hydroxyapatite substratum, showing some of the structural motifs found in *in vivo* plaque biofilm such as *Streptococcus* chains. (b) SEM (× 1510) of plaque biofilm grown in a CDFF, on a hydroxyapatite substratum, treated with an active Sonicare Plus® showing the bare surface of the disc with a small number of bacteria retained in depressions. This area of disc was proximal to the site of brushing. (c) SEM (× 5030) of plaque biofilm grown in a CDFF, on an untreated HA disc, representative of the predominant morphotype including intact *Streptococcus* chains. (d) SEM (× 5030) of HA disc treated with active Sonicare Plus® representative of the predominant morphotype including filamentous bacteria and individual cocci remaining after treatment with Sonicare Plus®. This area of biofilm was located away from the site of brushing. (e, f) CLSM projection image (159 × 159 µm: image depth = 76 µm) of untreated biofilm showing viable bacteria (e) and nonviable bacteria (f) from exactly the same location. (g, h) CLSM projection image (159 × 159 µm: image depth = 19.86 µm) of treated biofilm with an active Sonicare Plus® showing viable bacteria (g) and nonviable bacteria (h) from exactly the same location. Nonviable filamentous bacteria can be seen on the right-hand side of (h), suggesting that filamentous bacteria are susceptible to membrane damage by the shear forces produced by an active Sonicare Plus®.

Material und Methoden: Orale Biofilme mit multiplen Spezies, die aus menschlichem Speichel gewonnen wurden, wurden auf Hydroxylapatitscheiben in einem Fermenter für Filme von konstanter Dicke kultiviert. Die Biofilme wurden in ein Typodontmodell so platziert, dass sie approximale Plaque zwischen den Zähnen 46 und 47 simulierten und wurden dann mit der aktivierten bzw. nicht aktivierten elektrischen Zahnbürste behandelt. Die Distanz von den Borstenenden bis zum Rand der Scheiben betrug 2,65 mm. Die Bürstbewegung wurde durch eine speziell gebaute Bürstmaschine kontrolliert. Nach dem Bürsten wurde die Zahl der lebenden Bakterien, die aus den Biofilmen entfernt worden waren bzw. in ihnen verblieben, bestimmt.
Ergebnisse: 73,70% der lebenden Bakterien des Biofilms wurden mit der aktivierten Zahnbürste von den Scheiben entfernt. Eine nicht aktivierte Bürste entfernte nur 3,66%. Rasterelektronen- und konfokale Lasermikroskopie zeigten Unterschiede zwischen behandelten und unbehandelten Biofilmen.
Schlussfolgerung: Die Flüssigkeitsscherkräfte, die durch die elektrische Zahnbürste erzeugt wurden, reichten zumindest 2,65 mm über die Länge der Borsten hinaus und die Kräfte trugen zur Fähigkeit der Zahnbürste Plaque zu entfernen bei ($P < 0{,}001$).

Résumé

Effets de l'activité du fluide dynamique d'une brosse à dents électrique sur les biofilms buccaux in vitro
Le but de cette étude a été de déterminer l'efficacité à enlever la plaque dentaire d'une brosse à dents électrique Sonicare Plus® dans un modèle *in vitro*. Des biofilms buccaux avec plusieurs espèces provenant de la salive humaine ont été placés en culture sur des disques hydroxyapatite dans un fermentateur de films à profondeur constante. Les biofilms ont été placés dans un modèle typodont qui ressemble à la plaque interproximale entre les dents 46 et 47, et ont ensuite été traités avec une brosse à dents électrique tant activée que non-activée. La distance entre le bout des poils jusqu'au bord du disque était de 2,65 mm. L'action du brossage était contrôlée par une machine spécialement construite à cet effet. Après brossage, le nombre de bactéries viables enlevées et restantes des biofilms a été déterminé. Septante-quatre % des bactéries viables dans le biofilm ont été séparées des disques par la brosse activée. La brosse inactivée n'en enlevait que 4 %. Le microscope électronique à balayage et le microscope confocal ont révélé des différences entre biofilms traités et non-traités. Les forces de cisaillement du fluide générées par la brosse à dents électrique pénétraient au moins à 2,65 mm derrière la portée des poils et ces forces contribuaient à l'efficacité du brossage ($p < 0{,}001$).

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